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ORIGINAL INVESTIGATION

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Splice-site mutation in TGM1 in congenital recessive ichthyosis in American families: molecular, genetic, genealogic, and clinical studies

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Abstract Lamellar ichthyosis (LI, OMIM no. 242300) is a severe autosomal recessive genodermatosis with an estimated prevalence of 1:200,000. LI represents one end of the spectrum of congenital recessive ichthyosis (CRI). Mutations in the gene for transglutaminase-1 (*TGM1*) are responsible for many cases of LI and occur throughout the coding sequence of the gene. Our analyses of patients with CRI revealed a common *TGM1* mutation involving loss of the intron 5 splice acceptor site leading to alternative splicing of the message. We found families in which the splice acceptor site mutation was homozygous, and families where the patients were compound heterozygotes for the splice acceptor site mutation and another *TGM1* mutation. A mutation at this same site occurs in the majority of Norwegian patients as a founder effect. In our ethnically diverse patient population, none of whom have known Norwegian ancestry, haplotype analysis of the *TGM1* chromosomal region also suggested the existence of a founder effect. Comparison of the common haplotype in our data with the Norwegian data showed that 2/7 of our splice acceptor site mutation chromosomes had the full reported Norwegian haplotype, and the remaining five chromosomes exhibited recombination at the most distal marker studied. History, family origins, and haplotype analysis suggested that the mutation originally arose on a German background and was introduced into Norway around 800–1000 AD. We also found a limited correlation between genotype and phenotype in our study, with the four homozygous patients having less severe disease than many of the heterozygotes, and no patient with a splice acceptor site mutation having erythroderma or a congenital ichthyosiform erythroderma phenotype.

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Introduction

The congenital recessive ichthyoses (CRI) are a clinically and genetically heterogeneous group of disorders. The most severe, classic form of lamellar ichthyosis (LI, OMIM no. 242300) lies at one end of the spectrum of this group and usually presents at birth in the form of a collodion membrane covering the neonate and consisting of shiny taut skin that subsequently dries and peels away. Affected individuals then develop brown plate-like scale over the entire body. Ectropion, eclabium (turning outward of the eyelids and lips, respectively), and scarring alopecia (involving scalp and eyebrows) are frequently present. We and others have shown that mutations in the gene for transglutaminase-1 (*TGM1*) cause the vast majority of cases of classic LI (Russell et al. 1995; Huber et al. 1995). An exception is a group of seven families from Northern Africa, where the phenotype co-segregates with markers on chromosome 2, although no gene has yet been identified (Parmentier et al. 1996, 1999).

The other end of the spectrum of CRI is represented by those patients who, like patients with LI, often present as collodion babies and frequently also exhibit ectropion and alopecia, but who have erythroderma (red skin) and a fine white scale. This clinical presentation is termed congenital ichthyosiform erythroderma (CIE). It is still unclear if a subset, or even any, patients with CIE have mutations in *TGM1*.

Since we identified the underlying cause of LI, our laboratory has been interested in determining the range of *TGM1* mutations in the disease and in investigating the extent of correlation between clinical features and specific

gene mutations. During *TGM1* mutation screening of our CRI cohort, we identified the same mutation in several families. This single base substitution affected the canonical splice acceptor site between intron 5 and exon 6. Since our data set contained both affected individuals who were homozygous for the splice acceptor site mutation and those who were compound heterozygotes for the splice acceptor site and another *TGM1* mutation, we examined the contribution of the splice acceptor site mutation to the clinical presentation.

The splice-site mutation is the common *TGM1* mutation in CRI patients in the Norwegian population (Pigg et al. 1998). Limited analysis of very closely linked markers in these patients has suggested that there was a founder effect. A single German patient in this study was heterozygous for the splice-site mutation, but it was reported to be on a different chromosome 14q haplotype. The splice acceptor site mutation was also identified by Huber et al. (1995) in one presumably Swiss family. No genotyping of linked markers was presented, so it is not possible to determine whether these patients share a common haplotype with either the Norwegian patients or the single German patient.

Analysis of the transcription products of the splice-site mutation was reported in the Norwegians and in the Swiss patient. Interestingly, this mutation in the Swiss family produced an mRNA product that contained the complete intron 5 sequence inserted into the normal transcript, whereas this altered transcript was not described in the mRNA from the Norwegian patients. Instead, mRNA with a single G nucleotide inserted between exons 5 and 6 was reported. A mechanism for this insertion was not suggested, and the observation of two different mRNAs arising from the same genomic change has not been reconciled.

Here, we present results from five families with the intron 5/exon 6 splice acceptor site mutation. Affected members of two families were homozygous for the mutation, and those of the other families were compound heterozygotes. Haplotype construction identified a common founder chromosome spanning approximately 1.5 cM on chromosome 14q11. This haplotype shared many of the alleles of the Norwegian haplotype, although none of our patients claimed Norwegian ancestry. Genealogic information from our ethnically and racially diverse group of patients provided evidence that the founder chromosome may have arisen in the Westphalia region of Germany. We have observed the splice acceptor site mutation in association with those phenotypes at the LI end of the CRI spectrum. However, we have not observed the CIE phenotype in individuals having the splice acceptor site mutation. Among our patients, those individuals who were homozygotes for the splice acceptor site mutation were less severely affected than many of the compound heterozygotes. We also demonstrate the presence of both abnormal TGM1 mRNA transcripts in individuals carrying the splice acceptor site mutation and propose a mechanism for the appearance of the anomalous transcript with the inserted “G” originally noted in the Norwegian population.

Subjects and methods

Subjects

The families presented here were recruited for an ongoing study of the genetic basis of autosomal CRI. All patients and family members consented to participate in an IRB-approved protocol involving examination by a dermatologist and geneticist, clinical photography, collection of buccal swabs or blood for DNA, and skin punch biopsy. A detailed medical and dermatologic history, including the use of retinoids and other skin treatments, was obtained from all affected persons. Several patients were examined twice over the period of seven years during which the study was conducted, so that potential changes in phenotype could be evaluated.

mRNA isolation, cDNA synthesis, and genomic DNA preparation

Skin punch biopsies (2 mm) were homogenized in a Polytron tissue homogenizer (Brinkmann). Poly A+ mRNA was isolated with a Dynabeads mRNA Direct purification kit (Dynal) according to the manufacturer’s protocol and without elution. The mRNA bound to beads were then washed twice with 1×PCR buffer containing no $MgCl_2$, and first-strand cDNA was synthesized by extending the oligo(dT) primers attached to the beads (SuperScript Preamplification System; Life Technologies). The manufacturers protocol was modified by omitting the mRNA denaturation step at 70°C and by the addition of 1 μl of RNAsin (Promega) to the synthesis reaction. Genomic DNA was prepared from cells collected by buccal scraping, as previously reported (Richards et al. 1993).

Reverse transcription/polymerase chain reaction analysis

Reverse transcription/polymerase chain reaction (RT-PCR) was performed with cDNA Advantage 2 Polymerase mix (Clontech) and primers TGM1F and TGM1R by using a DNA Engine thermal cycler (MJ Research) and the following temperature profile: 94°C for 2 min, then 30 cycles of 94°C for 15 s, 61°C for 30 s, and 72°C for 3 min. The products were analyzed on a 0.8% agarose gel (SeaKem LE, FMC).

TGM1 fragment amplification

For sequence analysis of *TGM1* cDNA, five overlapping fragments were amplified with RedTaq DNA polymerase (Sigma) by using *TGM1* cDNA as the template and primer pairs having M13F or M13R sequences added to their 5’-ends (primers 3–12 in Table 1). PCR conditions were: 15 cycles of 94°C for 15 s, 63°C for 30 s, and 72°C for 45 s. Analysis of the aberrant amplification products resulting from the splice acceptor site mutation involved the use of primers TG1-F2 M13R and TGM1 SA5 M13F (Table 1).

Intron 5 splice acceptor mutation analysis

To assess the presence of the intron 5 splice acceptor site mutation in *TGM1*, genomic DNA was amplified with Platinum *Taq* DNA polymerase (Life Technologies) and primers TGM1mutF M13F or TGM1 SA5 M13F and TGM1mutR M13R (Table 1). PCR was performed under the following conditions: 94°C for 2 min, then 35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s. The products were analyzed on 2% agarose gel, and DNA fragments were cut out and purified by using the UltraClean Gel Spin kit (MoBio). The *TGM1* exon 5 splice acceptor site mutation was confirmed in affected individuals and carriers by *Msp*I digestion (Huber et al. 1995). The mutation created an additional *Msp*I restriction site, so that cleavage of the 467-bp PCR fragment generated from genomic DNA by using primers TGM1mutF and

Table 1 Sequences of primers used

	Primer name	Sequence	M13F tailed	M13R tailed
1	TGM1F	AACACTGCTGCCAGGCATGA	+	–
2	TGM1R	TCCGGGAGCCCTGGACTC	–	+
3	TGM1–1F M13F	ATGGATGGGCCACGTTC	+	–
4	TGM1–1R M13R	TGACCACCTGGGCTTTCC	–	+
5	TGM1–2F M13F	GCACGTGATCATCCCAGT	+	–
6	TGM1–2R M13R	TGGTGCCTCGGGAGTAA	–	+
7	TGM1–3F M13F	TGATTGGGAACTGGTCTGG	+	–
8	TGM1–3R M13R	CAATGAGTGTGCCGATGG	–	+
9	TGM1–4F M13F	TGGCAGCTTCAAGATTGTT	+	–
10	TGM1–4R M13R	ACCTGCCCGCTCTCCTT	–	+
11	TGM1–5F M13F	TACCGGCCCCATCTTGT	+	–
12	TGM1–5R M13R	GGACTCCCCACCTGAGC	–	+
13	TGM1mutF M13F	CCACCTCCGCCCTATCTC	–	+
14	TGM1mutR M13R	CCTGCCTGGCTCAGTCCTT	+	–
15	TGM1 SA5 M13F	ACTACGGGACCGAAGCACA	+	–

TGM1mutR without M13 extensions (Table 1) resulted in two fragments of 298 bp and 169 bp. The change in fragment size was visualized by agarose gel electrophoresis in 3% MetaPhor agarose (FMC).

Sequencing

In general, sequencing templates were prepared by electrophoresis in 2% agarose. The DNA bands visualized with ethidium bromide were excised and purified by using the UltraClean Gel Spin kit (MoBio). Simultaneous bi-directional sequencing of DNA templates (Roemer et al. 1997) was performed with the Sequitherm Excel II kit (Epicentre Technologies) and dye-labeled primers M13F IRD700 and M13R IRD800 (Li-Cor) as recommended by the manufacturers. Products were analyzed on a 4200S dual-dye automated sequencer (Li-Cor). Sequences were evaluated with Sequencher software (Gene Codes).

Genotyping

Genetic markers D14S1043, D14S72, D14S742, D14S581, D14S590, D14S264, TGM1.PCR1, D14S64, D14S1032, and D14S275 were typed on the Li-Cor 4200S by using 25-cm polyacrylamide gels. Sequences of the primers for genetic markers were obtained from GDB and modified so that one primer of each pair had a tail of either M13F or M13R. A 1-µl DNA sample was amplified in 5-µl reactions containing 0.1 pmol of each of the forward and reverse primers, 0.16 pmol of the corresponding fluorescently labeled M13 primer, 0.2 U AmpliTaq (Perkin-Elmer) in PCR buffer containing 2 mM $MgCl_2$ and 200 µM each dNTP. The amplification procedure consisted of an initial denaturation for 2 min at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 75 s, 72°C for 30 s, and a final extension for 5 min at 72°C. To insure that our allele assignments corresponded to those of the Norwegian study of Pigg et al. (1998), we also genotyped the DNA of eight individuals used in that study (a generous gift of M. Pigg and T. Gedde-Dahl).

Haplotype analysis

The frequency of the specific allele associated with the conserved haplotype at each locus was compared with the frequency of that allele on 14 chromosomes (three carrying other *TGM1* mutations in the families, plus eleven normal chromosomes segregating in the families). Assuming no linkage disequilibrium among the markers typed (i.e., the frequency of any specific allele at a marker locus was independent of the allele at any of the other loci), we calculated the frequency of each marker allele on the mutation-carrying haplotype and the control haplotypes. These frequencies were compared with published allele frequencies (when available) and found to be similar.

Results

Clinical studies

Nine persons with congenital recessive ichthyosis and a mutation in the intron 5/exon 6 canonical splice site in TGM1 were identified in five families (Fig. 1). The clinical features of the affected individuals are summarized in Table 2. All affected persons had congenital onset of the disease with a history of collodion presentation and heat intolerance. None of the patients were erythrodermic when not on retinoid therapy. Family I015 included three affected siblings and their unaffected mother. The deceased father was also reportedly unaffected. The sisters, II-1 and II-3, were in their 40s when first examined by us. Both exhibited a similar phenotype, with severe alopecia of the scalp, palm and sole hyperkeratosis, and generalized light brown scale. They showed attenuation of the features of LI and the generalized erythroderma associated with their oral retinoid and vitamin A therapy (Fig. 2, right column). Their brother (II-2) was more mildly affected than his sisters and used only mild keratolytics to control the scaling.

Family I020 has been reported previously (Huber et al. 1997). The proband in the family was individual III-4, who was first examined by us at age 7 years and was affected with white scale with accentuation over the flexures, mild alopecia of the scalp, and mild hyperkeratosis of the palms and soles. Upon re-examination at age 14 years, he showed an excellent response to topical use of alpha- and poly-hydroxy acids. This three-generation family also consisted of a grandfather (I-1) and grandson (III-1) who did not have congenital ichthyosis but were affected with mild scaling localized to the dorsum of the

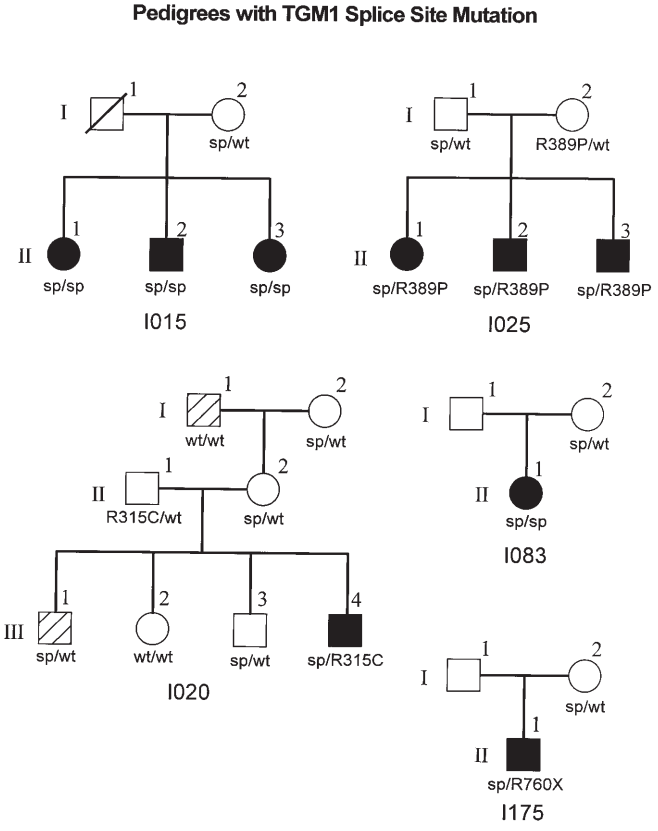

Fig. 1 Pedigrees of the five families with the splice-site mutation. *Filled symbols* Individual with congenital recessive ichthyosis, *hatched symbols* individuals in family I020 with ichthyosis-like phenotype, not of congenital onset, *sp* splice-site mutations, *wt* wild-type sequence, *R389P, R315C, R760X* other *TGM1* mutations

foot and the extensor surfaces of the upper and lower extremities. The findings in I-1 and III-1 were consistent with an ichthyosis-vulgaris-like phenotype, although II-2 had normal skin.

There were three affected siblings in family I025, and the parents were unaffected and unrelated. Individuals II-1 and II-2 had generalized plate-like scale with thickening of the palms and soles; II-1 also had decreased eyelashes and eyebrows and increased thickening of the palms with increased age. In addition, II-2 exhibited scarring alopecia of the scalp. Individual II-3 had generalized scale when seen at age 2 years.

Family I083 consisted of a 6-year-old girl and her unaffected mother. The father was reportedly unaffected and not related to the mother. The proband exhibited generalized yellow-brown plate-like scale, ectropion, alopecia of the scalp, and hyperkeratotic palms and soles (Fig. 2, middle column). Re-examination at age 9 years revealed a good response to topical application of alpha- and polyhydroxy acids.

The proband in family I175 was a 9-year-old African American male. His unaffected mother and reportedly unaffected (and unavailable) father were not related. The boy had mild eclabium, severe ectropion, thick palms and soles, and generalized thick dark plate-like scale over the entire body. He also had severe scalp involvement with alopecia. His appearance defines the phenotype of severe classic LI (Fig. 2, left column).

Mutation analyses

Four of the patients with CRI in this study were homozygous for a change of A to G at position 2526 in genomic DNA (three in family I015 and one in family I083), whereas the remaining five patients in three families were compound heterozygotes. In those patients heterozygous for the splice mutation, we also determined the complementing mutation of the other allele. In one case, a mutation causing a change Arg to Cys at residue 315 was identified (previously reported by Huber et al. 1997). We also identified a *TGM1* allele coding for an Arg to Pro (CGC to CCC) mutation at residue 389, and an allele bearing an Arg to Stop (CGA TO TGA) change at amino acid position 760. Results are noted in the pedigrees in Fig. 1. Several polymorphisms in DNA sequence were observed in unaffected individuals from different families: Lys152Lys (AAG/AAC), Met665Met (ATG/ATC), Pro352Pro (CCA/CCC), and T2548C in the 3' untranslated sequence.

The A to G mutation in the splice acceptor of intron 5 has been reported to have two different consequences on TGM1 mRNA processing. In one study, transcripts retaining intron 5 were described (Huber et al. 1997), whereas in a second study, transcripts with only a single G nucleotide inserted between exons 5 and 6 were noted (Pigg

Table 2 Clinical features of individuals affected with the splice-site mutation in TGM1 (*PG* propylene glycol, *U* urea, *P* prednisone, *VA* oral vitamin A, *tRA* topical retinoic acid, *I* isotretinoin, *LA* lactic acid, *AHA* alpha hydroxy acid, *GA* glycolic acid)

Case no.	Age at examination	Ectropion	Eclabium	Palmo-plantar hyperkeratosis	Alopecia	Plate-like scale	Treatment history
I015-II-1	48, 55	–	–	+	+	–	PG, U, P, VA, tT
I015-II-2	45	–	–	–	–	–	PG, LA
I015-II-3	43, 50	–	–	+	+	–	I, LA, AHA, VA, tT
I020-III-4	7, 14	+	–	+	+	+	LA, GA, AHA
I025-II-1	5, 10	+	–	+	–	+	PG, tT
I025-II-2	4	+	–	+	+	+	PG, tT
I025-II-3	2	+	–	+	–	–	PG, tT
I083-II-1	7, 10	+	–	+	+	+	PG, LA, AHA
I175-II-1	9	+	+	+	+	+	PG, LA, tT

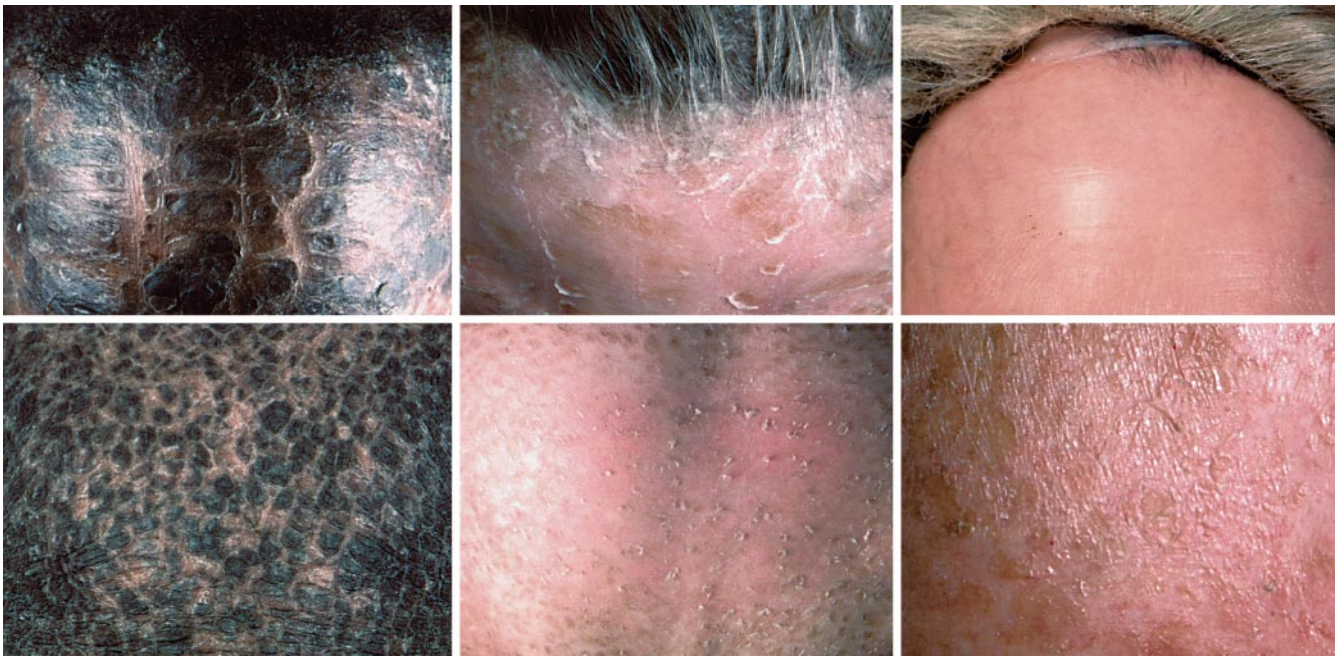

Fig. 2 *Top row* Forehead of three patients, *bottom row* mid-back of three patients. *Left column* I175-II-1, 9-year-old male, compound heterozygote for splice-site mutation and R760X showing generalized large plate-like dark scale, and alopecia (over the periphery of the frontal and temporal scalp). *Middle column* I083-II-1, 6-year-old female, homozygous for the splice-site mutation, with generalized light brown, thin, scale and mild alopecia over the periphery of the scalp. The slight redness is an artifact of the photographic processing; she was not erythrodermic. *Right column* I1015-II-1, 48-year-old female, homozygous for the splice-site mutation, with accentuated yellow scale, and severe alopecia

et al. 1998). Both of these variant transcripts predict premature termination of TGM-1 protein translation from an in-frame stop codon, 43 codons or 1 codon downstream from the last residue of exon 5, respectively. To address this discrepancy, we amplified TGM1 cDNA by using primers in exons 5 and 6 (primers 6 and 15 in Table 1) from affected, carrier, and normal individuals and analyzed the resulting products. Two DNA bands were observed in agarose gels in both homozygous and heterozygous individuals (Fig. 3), but only the smaller fragment was seen in normal controls. Sequencing confirmed that the larger fragment corresponded to an mRNA with intron 5 inserted as predicted between exons 5 and 6 (353 bp). In these homozygous individuals, the sequence of the smaller fragment revealed only a single G nucleotide inserted between the two exons. The amount of the smaller fragment was estimated to represent less than 5% of the amount of the larger fragment (Fig. 3, lanes 1, 4). In heterozygotes, this splice variant could not be detected by sequencing. This was not unexpected, since sequencing is not an appropriate method for detecting the presence of the extra-G sequence predicted to be present at only about 2.5% that of the normal sequence. Using a more sensitive assay based on selective amplification of the extra-G sequence with a sequence-specific primer, we demonstrated the presence of this aberrant transcript in heterozygous and in homozygous individuals, but not in individuals who did not carry the splice acceptor site mutation (data not shown).

Evidence of a founder effect

The ethnic and racial compositions of the five families with the splice acceptor site mutation were quite diverse. Both parents of the three affected individuals in family I015 were born in the Midwestern United States in the early 1920s. The four grandparents of the affecteds, however, were from the same region of Germany, known as Westphalia. The grandparent (I-2) who introduced the

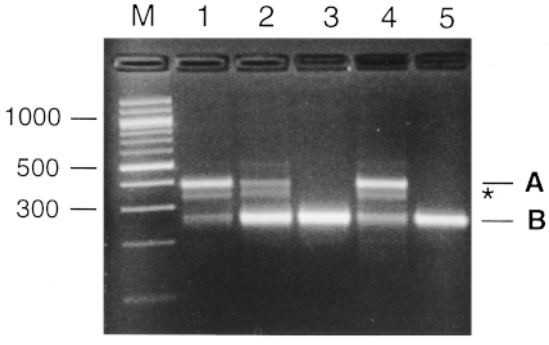

Fig. 3 The intron 5 splice acceptor site mutation leads to two alternative TGM1 mRNA splice variants. The lanes represent RT-PCR analysis of TGM1 mRNA from the epidermis of homozygotes (*lanes 1, 4*), a heterozygote carrier (*lane 2*), a non-carrier sibling (*lane 3*), and an unrelated normal individual (*lane 5*), and markers (*M*). Amplification with primers 6 and 15 (Table 1) located in exon 5 and exon 6 yielded a large fragment containing intron 5 (*A*, 392 bp) and a small fragment (*B*, about 264 bp). The third band (*) represents a probable heteroduplex between the two amplimers

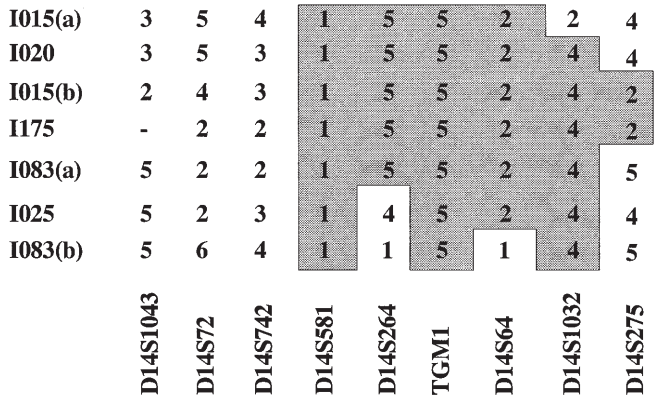

Fig. 4 Haplotypes carrying the TGM1 splice-site mutation. Patients in families I015 and I083 were homozygous for the splice-site mutation; therefore, the haplotype of each allele is shown separately (*a, b*). The shared haplotype is shaded in *gray*

splice acceptor site mutation into family I020 was also of German ancestry, although the specific region of origin was unknown. The ancestry of the splice acceptor site mutation carrier mother in family I083 was stated as German and Irish. Family I175 was African American. No patients claimed any Norwegian ancestors, and as most Norwegians emigrated to the United States around the turn of the last century, it is likely that their descendants would be aware of this family history. Information about ethnicity was unavailable for I025, as the family had been in the United States for many generations.

The presence of the identical mutation in these five families led us to test the hypothesis that this mutation arose on a founder chromosome in the distant past. We analyzed nine polymorphic genetic markers encompassing approximately 16 cM in and around the *TGM1* gene and constructed haplotypes for the mutant and normal *TGM1* chromosomes in the families. A conserved haplotype encompassing the markers D14S581, D14S264, TGM1, D14S64, and D14S1032 could be identified (Fig. 4). Using the allele frequencies calculated from control chromosomes, we found that the probability that the canonical conserved haplotype carrying the splice acceptor site mutation had occurred at random in the population was 0.00028. The observed frequency of this haplotype, however, was 0.5436, a difference of greater than 1900 times. The greatest contribution to this difference was attributable to the D14S64 locus. Six of the seven splice acceptor site mutation chromosomes carried the "2" allele at this locus, whereas none of the eight normal chromosomes and only one of the three chromosomes carrying other *TGM1* point mutations had a "2" allele at this locus. In contrast, there was no significant difference in allele distribution between the splice acceptor site mutation chromosomes and the control group at the loci flanking the conserved region (D14S742, D14S275).

We compared the conserved haplotype in our set of families with that reported in the Norwegian study, and with the single previously reported German patient with a splice-site mutation. All of our splice-mutation-carrying chromosomes carried the same *TGM1* allele, as did all 70 of the Norwegian patients, whereas this allele occurred on 27% of our control chromosomes. In addition, the conserved D14S264/D14S64 haplotype was the same in both our data and the Norwegian data. We also typed D14S1032 (distal to D14S64) and found that 6/7 of the splice-site-mutation-carrying chromosomes in our data had the "4" allele (compared with 18% of controls). This marker was not typed in the Norwegian patients. Finally, 2/7 chromosomes in our data had the "2" allele at D14S275, as did one-third of the Norwegian chromosomes. The single German haplotype reported by Pigg et al. (1998) was the same only at the T*GM1* and, possibly, the D14S64 loci.

Discussion

Whereas most mutations in *TGM1* have been found in only one or a few families, we have identified a common *TGM1* mutation involving the loss of the exon 5 splice acceptor site in LI patients of various ethnic and racial backgrounds. Although this mutation had been described previously, it was thought to be virtually limited to the Norwegian population. Our identification of the mutation in patients of non-Norwegian ancestry prompted an analysis of the haplotypes on which the mutation was carried. To our surprise, a common haplotype encompassing at least 1.5 cM around the *TGM1* gene was common among our patients and also was shared with the Norwegian patients. Family histories from our five families suggested that the mutation may have arisen in Germany, as at least five of the ancestors were German, and four were from the same region in Germany known as Westphalia. The single African American patient may well have had a distant German ancestor, as it has been calculated that approximately 30% of the genes in American blacks are attributable to Caucasian-African racial intermixture (Glass and Li 1953). Indeed, immigration of Germans to the United States occurred as early as 1608, with concentrations of Germans in Philadelphia, New York, Missouri, Virginia, Georgia, and the Carolinas. Some were certainly slave holders, including the German Quakers, until the mid 1700s. Illicit relationships between white slave holders and black slaves was one manner in which Caucasian genes were introduced into the African American population.

More difficult to understand is the relationship between the Germans and the Norwegians with respect to this *TGM1* mutation. The age of the splice acceptor site mutation can be estimated from the size of the conserved haplotype surrounding it. If one assumes that the conserved haplotype is minimally from *TGM1* to D14S275 (as presented by Pigg et al. 1998), the size of the conserved region is approximately 1.5 cM. However, the Norwegian haplotypes were not extended proximal to the TGM1 locus. In our data, we have found that the "1" allele is completely conserved at D14S581, extending the haplotype to approximately 3.0 cM. If we also hypothe-

size that the Norwegian population of LI patients descended from a small group or even a single founder, and that the population expanded at a constant exponential rate, we can apply the Luria-Delbruck method (Luria and Delbruck 1943) to estimate the number of generations (g) that has passed since this mutation was introduced into the population, i.e., $1–e^{-g(\text{theta})}$ equals the proportion of chromosomes carrying the mutation in which recombination with the conserved haplotype has occurred. In our data, 5/7 haplotypes have recombined with D14S275 (do not carry the "2" allele at this locus) and, in the Norwegian data, 19 of 68 chromosomes carry the "2" allele. Both these data sets give estimates that recombination with the ancestral chromosome has occurred in approximately 72% of the cases. Solving for g, the introduction of the mutation to the Norwegians occurred approximately 45 generations ago. If a generation spans 20 years, this mutation would have been introduced around 1000–1100 AD. Over a lengthy period of time, but starting around 800 AD, Christianity was introduced into Norway. Missionaries from the churches of several countries, including Germany, were responsible for weakening the traditional belief in the Nordic gods. It is possible that a missionary brought the splice acceptor site mutation in *TGM1* and introduced it to the population. In addition, our family data suggest that the region of Germany in which the mutation derived may have been Westphalia, along the Northern Rhine, since four of the mutation-carrying grandparents immigrated to the United States from this area.

The Norwegian population underwent a significant bottleneck in 1350 when the bubonic plague, known as the Black Death, reduced the population to one-half or one-third of its pre-1350 level. This historical event may account for the high frequency of this gene in the population, with the incidence of LI and CIE in Norway being reported at 1/91,000 over the last 10 years (Pigg et al. 1998). This is more than twice the frequency estimated in the US population (1:200,000–300,000; Bale and Doyle 1994).

After identifying the splice-site mutation in the small series of patients presented here, we went back to our total collection of banked genomic DNA samples from CRI families and used a restriction enzyme analysis to screen for the mutation. Of 47 families from whom DNA was available, we identified two more families who carried the mutation, bringing to five the number of families in which the splice mutation was heterozygous, along with the two homozygous families. This gives a frequency of 9.6% for the splice mutation allele in our dataset (9 splice-mutation alleles/94 alleles tested). However, this is a very heterogeneous group of families, both clinically and ethnically, and no conclusions about the frequency of this mutation in LI can be drawn directly from these data.

The mutation in the Norwegian families was reported to result in an insertion of a G in the resultant mRNA (876insG). This splice-site mutation was also identified by Huber et al. (1995) in one Swiss family, but in that family, the same A to G substitution was reported to result in an mRNA product that retained the complete intron 5 sequence in the normal transcript. In contrast, we found that mRNA from our patients consisted of two transcripts. One with intron 5 inserted at the exon 5 splice represented approximately 90% of the transcripts, whereas the other mRNA fragment with the inserted G represented the remaining 10%. Failure of the spliceosome to excise intron 5 is consistent with the loss of the canonical AG sequence caused by the splice acceptor mutation. A mechanism for the formation of the extra-G transcript can be proposed based on an examination of the sequence at the 3'-end of intron 5 of the mutated TGM1, ..**ccctccttccg**/**G**GTTT... In order to retain an extra G but excise the remainder of intron 5, we suggest that the sequence shown in bold functions as a splice acceptor, with cleavage occurring as indicated. This sequence corresponds to the extended "consensus" splice acceptor recognition site identified by "logo" analysis **(**Stephens and Schneider 1992**)**, with the significant exception of the penultimate "g" (normally an "a"). We suggest that this variant sequence supports inefficient, but still appreciable, spliceosome activity.

Whether CIE can be caused by mutation in *TGM1* is still a matter of controversy. Many of the "CIE" patients in whom *TGM1* mutations have been reported to date were evaluated while on retinoid treatment (Pigg et al. 1998) or treatment information was not presented (Hennies et al. 1998; Laiho et al. 1997), so that the true clinical diagnosis may have been masked. Classification of the Norwegian patients was based primarily on electron-microscopic findings (Niemi et al. 1994; Arnold et al. 1988; Anton-Lamprecht 1992), history, and clinical evaluation. Thirty-three patients were homozygous for the splice-site mutation. Of these, 29 had the LI phenotype, one appeared to have CIE at birth but developed an LI phenotype later in life, two had a CIE phenotype, and one had a CIE phenotype but was known to be on retinoid therapy at the time of evaluation. The authors thus concluded that both CIE and LI can be caused by the same mutation.

In this study, we obtained detailed clinical information about each of the patients, including topical treatment and oral medication history, with particular attention to retinoid use. Therapy with systemic retinoids causes desquamation of thick abnormal stratum corneum. In patients with LI, loss of the thick scale does not leave a normal epidermis; rather, the resulting epidermis is usually covered with a fine scale and is commonly prone to erythema. In our study, we found that none of the patients, either homozygous or heterozygous for the splice acceptor site mutation, were erythrodermic, except when using retinoid therapy. Although we did not note any specific clinical variables that could be directly attributed to the splice-site mutation, and the clinical manifestations ranged from the most severe LI phenotype to a milder phenotype without ectropion, eclabium, or typical plate-like scale, none of these patients had CIE.

To continue to facilitate investigations into genotype/phenotype correlations in the recessive ichthyoses, it is imperative to continue to collect and to present data on the therapeutic history of the patients. This is only in this way

that it will be possible to resolve the genetic complexity of these clinically heterogeneous disorders.

Note added in proof. An analysis of the effect of the A2526G mutation on splice-site acceptor site quality was performed by James Ellis, National Institutes of Health, using an implementation of the Delila algorithms developed by Tom Schneider (National Institutes of Health). The results confirm our speculation concerning inefficient cryptic splice acceptor activity on mRNA from the mutant TGM1 allele. As expected, the original strong acceptor site was found to be highly degraded by the mutation (from a score of 12.8 bits to 6.4 bits), and a moderately strong acceptor was predicted at position 2525 (6.7 bits). An acceptor site of this quality would result in residual splicing of the normal type, perhaps up to 1/4 as strong as the "frame-shifted" splicing responsible for transcripts having the G insertion.

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